AD_____

Award Number: DAMD17-03-1-0016

TITLE: Toward a Diagnostic Immunoassay Specific for Prostate Cancer: Chemical Synthesis of Homogeneous N-Linked Prostate Specific Antigen Glycopeptides

PRINCIPAL INVESTIGATOR: Justin S. Miller, Ph.D.

CONTRACTING ORGANIZATION: Sloan-Kettering Institute for Cancer Research
New York, NY 10021

REPORT DATE: March 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2004	Annual (1 Mar 2003 - 28 Feb 2004)

4. TITLE AND SUBTITLE
Toward a Diagnostic Immunoassay Specific for Prostate Cancer: Chemical Synthesis of Homogeneous N-Linked Prostate Specific Antigen Glycopeptides

5. FUNDING NUMBERS
DAMD17-03-1-0016

6. AUTHOR(S)
Justin S. Miller, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Sloan-Kettering Institute for
Cancer Research
New York, NY 10021

E-Mail: miller7@mskcc.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Current prostate cancer (PCa) diagnostic tools are unsatisfactory because of their inability to determine the root cause of elevated levels of prostate specific antigen (PSA), which can arise from benign or malignant conditions. The purpose of the research described is the development of an improved PCa immunoassay based on errant PSA glycoform expression in prostatic cancer cells. Specifically, the aim is to define and implement a strategy for the synthesis of normal and transformed PSA glycopeptides, and to employ the PSA glycopeptides in immunological studies targeting the generation of antibodies that differentiate between normal and cancerous PSA. Such differentiating antibodies would comprise the sought-after improved PCa diagnostic. The first round of synthetic studies is complete: a strategy has been defined for the chemical synthesis of complex glycans and glycopeptides, including PSA-based glycopeptides. The synthetic strategy has been applied successfully to the syntheses of normal and transformed fragments of PSA. A collaboration is currently underway, during which the PSA glycopeptides will be conjugated with a carrier protein (keyhole limpet hemocyanin, KLH), and attempts will be made to generate antibodies that can differentiate between normal and transformed glycoforms of PSA.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Prostate specific antigen, PSA, prostate, cancer, PCa, diagnosis, immunoassay, glycopeptide, synthesis, glycoform	17
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Diagnostic tools for prostate cancer (PCa) have improved over the last decade with the use of prostate specific antigen (PSA) as a marker for the disease. PSA is a 237-residue glycoprotein secreted by the prostatic epithelium. The gross serum level of PSA was originally used as a key cancer diagnostic, but assays based solely on PSA levels cannot distinguish between patients with PCa and those with benign prostatic hyperplasia (BPH) at PSA serum levels between 4 and 10 μg/L. It was later found that patients with BPH displayed elevated levels of free PSA relative to their total amount of serum PSA; combinations of the original assay and the new, comparative assay of free to total PSA were reported to yield more accurate diagnoses, but the utility of such assays remains under debate. Another method for PCa diagnosis based on serum PSA content, called PSA velocity, involves monitoring increased PSA levels over time for a particular patient, but this method is prone to errors as it necessitates accurate concentration measurements over large time intervals. Thus prostate cancer diagnosis would benefit from a new, more accurate immunoassay.

The structure of PSA consists of a polypeptide backbone with an *N*-linked carbohydrate moiety. It has been shown that metastatic prostate cancer cells express larger, more highly branched carbohydrates than do normal prostate cells. The differentially glycosylated region of transformed PSA could be used as a molecular marker specific for PCa over BPH. Study of this issue in detail to develop a new PCa immunoassay requires pure, homogeneous PSA glycopeptides, but useful samples of homogeneous glycosylated PSA from natural sources are prohibitively difficult to obtain. Homogeneous PSA-derived glycopeptides may be secured, however, through chemical synthesis.

The research described below at its outset targets a paradigm for the total synthesis of *N*-linked PSA glycopeptides; the ultimate goal is a more accurate assay for prostate cancer. Our synthetic objectives are to: (1) design and carry out a method for glycan synthesis that is easily modified to incorporate higher degrees of carbohydrate branching; (2) incorporate synthetic glycans into relatively long PSA peptides using a fast, high-yielding strategy that remains synthetically flexible; and (3) optimize the glycopeptide structures based on their abilities to generate antibodies for use in an immunoassay while retaining the glycan features that distinguish cancerous PSA from normal PSA.

Research Update

The first two objectives have been realized, while progress toward the third is in the initial stages. Fulfilling the first objective, a strategy was designed for oligosaccharide synthesis that allows a single intermediate (Figure 1) to function as a synthetic template upon which all of the required PSA glycans—indeed, upon which any *N*-linked glycan—can be generated. Details regarding the application of precursor **1** towards syntheses of the *N*-linked trisaccharide[1] and pentasaccharide[2] cores, and of normal and transformed PSA glycans[3] can be found in the appended publications.

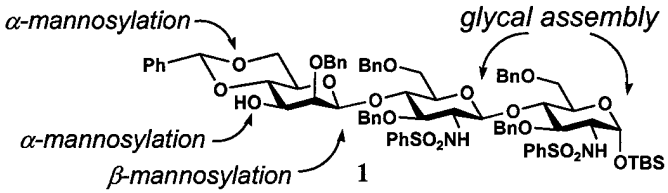


Figure 1. A common precursor that has been employed for the synthesis of various *N*-linked carbohydrates.

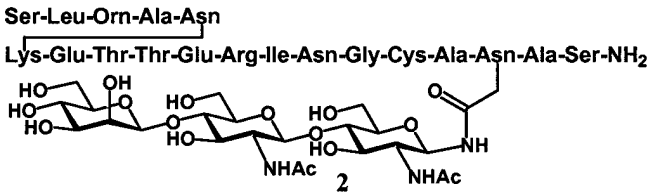
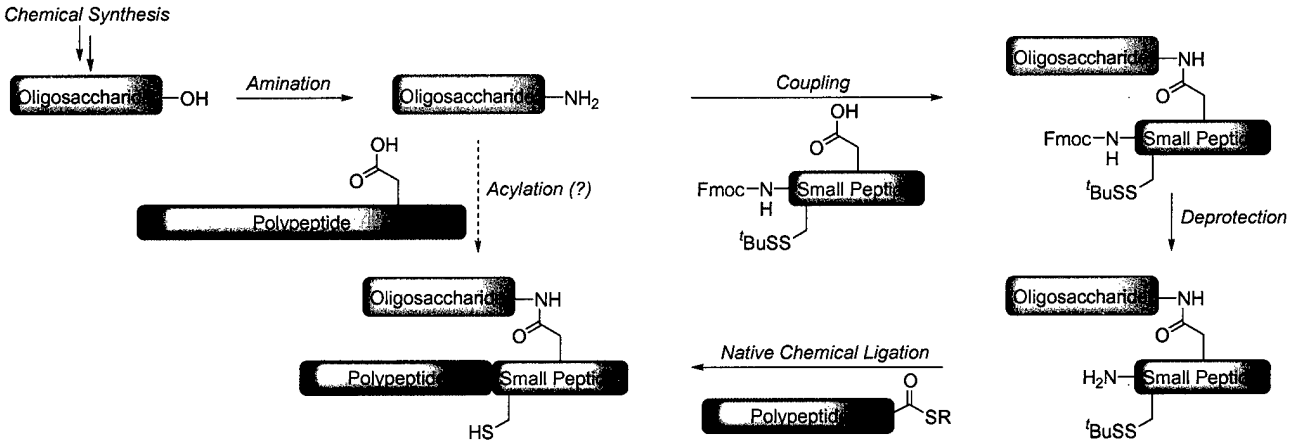


Figure 2. Glycopeptide synthesized during model studies. Details of these studies can be found in the appended publications.[1]

The second objective of glycopeptide synthesis was approached first through a model study that delivered trisaccharide glycopeptide **2**, presented in Figure 2. The synthetic strategy employed a new combination of methods that permits access to fully functional *N*-linked glycopeptides of significant peptide length and, in principle, unbounded glycan diversity. Depicted in Scheme 1, the method involves chemical synthesis of the free saccharide, conversion of the anomeric hydroxyl to a β-amino function, acylation with an aspartate-containing peptide, and elongation of the peptide *via* native chemical ligation. A full account of this model study, including all of the appropriate references, may be found in the appendix.[1]



Scheme 1. A representation of the methodology developed for and employed in the synthesis of model and PSA glycopeptides.[1]

The synthetic scheme shown in Scheme 1 paved the way for the syntheses of the normal and transformed PSA glycopeptides illustrated in Figure 3. The normal (black color only) and transformed PSA fragments were constructed in the same concise manner as was model glycopeptide **2** above, which is particularly significant given the unprecedented complexity of the synthetic targets, both in terms of the carbohydrates themselves and in terms of the entire glycopeptide constructs. A description of these completed syntheses has been published, and is included as part of the appendix;[3] the details are therefore not discussed further here.

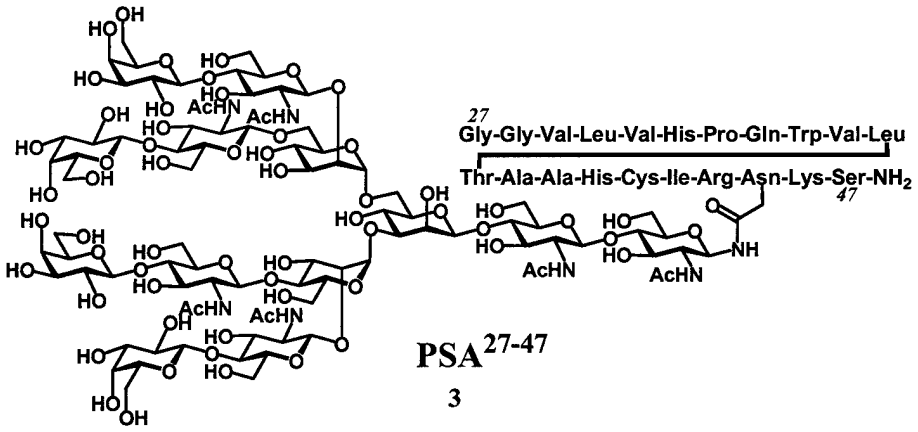


Figure 3. Successfully synthesized normal (black) and transformed (black and blue, or black, red, and blue) PSA glycopeptides.[3]

The third goal—glycopeptide structure optimization for antibody generation—will be facilitated by the design principles employed in accomplishing objectives (1) and (2). The convergence inherent in the synthetic approach permits relatively effortless variation of the target glycopeptide structures during the next phase of research, to be performed in collaboration with the Laboratory for Molecular Pharmacology at the Sloan-

Kettering Institute. Covalent conjugation of the PSA glycopeptides to an immunostimulatory agent might involve, for example, standard coupling of the Cys^{42} thiol with a maleimide-modified carrier protein, such as keyhole limpet hemocyanin (KLH); these experiments are already underway. If this particular technique for antibody generation proves unsuccessful in producing the desired results due to an insufficient immune response, the modularity of the glycopeptide synthesis easily allows the incorporation of other immunostimulants or adjuvants that might resolve the problem. For example, Pam_3Cys can be incorporated covalently, late in the synthesis, as part of the peptide sequence; QS21 can be coadministered during immunization with the PSA glycopeptides; or both could be employed in concert, depending on which combination is most effective at generating useful antibodies. It is important to note that experiments involving live animal subjects will be performed under the auspices of the Laboratory for Molecular Pharmacology at the Sloan-Kettering Institute, and will be funded independently of this grant.

Summary

In summary, the first two synthetic objectives of this research have been achieved, and experiments are underway that are directed at the third and final goal, which entails generating antibodies capable of differentiating between normal and transformed glycoforms of PSA. Completed syntheses of the normal and transformed PSA-derived glycopeptides, which as synthetic targets exhibit unprecedented glycan and glycopeptide complexity, represent a milestone in this research. Their successful achievement constitutes a significant advance toward the ultimate goal of an improved prostate cancer diagnostic tool.

Training Experience

The collegial atmosphere in the Danishefsky laboratory at the Sloan-Kettering Institute for Cancer Research has offered an excellent setting in which to broaden my chemical, biological, and immunological knowledge base. Interactions with fellow chemists have provided me with an appreciation for, and background in, carbohydrate synthesis. Collaborations with biologists and immunologists, particularly in the Muir group at Rockefeller University and in the Scheinberg group at the Sloan-Kettering Institute, have introduced me to aspects of biomedical research that might have eluded me in a typical academic setting. Issues have been brought to my attention regarding peptide bioconjugate synthesis, both in the abstract and as applied to practical questions, and I have been introduced to biological aspects of carbohydrate, peptide, protein, and glycoprotein immunogenicity. Through this funding opportunity I have also garnered an enhanced appreciation for the biochemical challenges associated with prostate cancer diagnosis, progression, and treatment. Along with frequent and wide-ranging discussions with Dr. Danishefsky, these experiences will prove invaluable as I move forward in my career and develop an independent research program that focuses in part on cancer-related issues.

Statement of Work (reproduced from the original submission)

Objective 1: Completion of the Model Study
Task 1: Month 1: Preparation of glycosylamine from ["normal" oligosaccharide]
Task 2: Month 2: Model glycosylation and native chemical ligation (NCL) with the nonasaccharide glycopeptide

Objective 2: A Normal PSA Glycopeptide
Task 3: Months 3-4: Synthesis of PSA short peptide; optimize glycosylation, protecting group strategy
Task 4: Months 5-6: Synthesis of PSA thioester, optimize NCL conditions
Task 5: Month 7: Antibody generation with a normal PSA glycopeptide*

Objective 3: A "Transformed" PSA Glycopeptide
Task 6: Months 8-12: Synthesis of a potential "transformed" PSA glycan
Task 7: Months 13-14: Glycosylation, NCL
Task 8: Month 15: Antibody generation with a "transformed" PSA glycopeptide*

Objective 5: Optimization of Glycopeptide Immunogenicity
Task 9: Months 16-20: Synthesis of structural variants of normal and "transformed" PSA glycopeptides, including covalently bound adjuvants, for antibody generation*
Task 10: Months 21-24: Optimize the structure of PSA glycopeptides for the generation of antibodies*

***Funding for antibody generation using live animal subjects is not being requested under this proposal; all such experiments will be performed under the auspices of the Laboratory for Molecular Pharmacology at the Sloan-Kettering Institute, and will be funded independently of this grant.**

The first three Objectives (Tasks 1-8) outlined in the approved Statement of Work (reproduced above), which were scheduled for completion after 15 months, rather than 12 months, have been largely accomplished. The notable exceptions are Tasks 5 and 8, which both involve antibody generation in collaboration with the Laboratory for Molecular Pharmacology at the Sloan-Kettering Institute. While these experiments are only just underway, both the early completion of the first round of synthetic Tasks and the fact that Task 8 has been initiated several months ahead of schedule indicate appropriate progress regarding the Statement of Work.

Key Accomplishments

- Development of a broadly applicable strategy for *N*-linked carbohydrate synthesis
- Development of a broadly applicable strategy for *N*-linked glycopeptide synthesis
- Demonstrations of the aforementioned synthetic strategies in model and pertinent systems
- Synthesis of a "normal" PSA glycopeptide
- Synthesis of two "transformed" PSA glycopeptides
- Initiation of a collaboration aimed at generating antibodies specific to normal and transformed glycoforms of PSA

Reportable Outcomes

Published Manuscripts

(1) Miller, J. S., Dudkin, V. Y., Lyon, G. J., Muir, T. W., and Danishefsky, S. J. "Toward Fully Synthetic *N*-Linked Glycoproteins." *Angew. Chem. Int. Ed.* **2003**, *42(4)*, 431-434, and references therein.

(2) Dudkin, V. Y., Miller, J. S., and Danishefsky, S. J. "A Concise Route to the Core Pentasaccharide of *N*-Linked Glycoproteins." *Tetrahedron Lett.* **2003**, *44(9)*, 1791-1793, and references therein.

(3) Dudkin, V. Y., Miller, J. S., and Danishefsky, S. J. "Chemical Synthesis of Normal and Transformed PSA Glycopeptides." *J. Am. Chem. Soc.* **2004**, *126*, 736-738, and references therein.

Patents

(1) Danishefsky, S. J.; Miller, J. S.; Dudkin, V. Y. "Prostate Specific Antigens, Conjugates Thereof, Methods for their Preparation and Uses Thereof." PCT application, filed **2003**.

Employment Opportunities

Based in part on the experience and training supported by this award, the primary investigator (PI) applied for and was awarded a tenure-track position of Assistant Professor in the Department of Chemistry at Hobart and William Smith Colleges in Geneva, NY. The position requires excellence in teaching and mentorship of undergraduates both in the classroom and in the laboratory. Responsibilities include maintaining an active research program staffed by undergraduates, along with teaching duties.

During the employment application process, the research supported by this award was presented at a total of eight academic (undergraduate and also graduate) institutions across the country.

References

The citations included in the published manuscripts (see citations above; full text in Appendices A-C) serve as a complete references section; a separate such section is therefore not included here.

Coordination polymer **2** was synthesized according to a published procedure. Elemental analysis (%) calcd for $C_{13}H_8NO_4Cu$: C 51.06, H 2.62, N 4.58; found C 50.62, H 2.45, N 4.60.

Gas adsorption measurements: Sorption isotherms were measured at 298 K on an FMS-BG (BEL inc.) automatic gravimetric adsorption measurement system with Rubotherm magnet coupling balance incorporated in a SUS steel pressure chamber. A known weight (200–300 mg) of the as-synthesized sample was placed in the aluminum sample cell in the chamber, and the sample was dried under high vacuum at 373 K for 5 h to remove the host water molecules. The adsorbate was dosed into the chamber, and the change in weight was monitored. After correction for buoyancy, the absorbed amount was determined.

Received: August 27, 2002 [Z50052]

[1] G. A. Ozin, A. Kuperman, A. Stein, *Angew. Chem.* **1989**, *101*, 373–390; *Angew. Chem. Int. Ed. Engl.* **1989**, *28*, 359–376.
[2] A. Corma, *Chem. Rev.* **1997**, *97*, 2373–2419.
[3] M. Eddaoudi, D. B. Moler, H. Li, B. Chen, T. M. Reineke, M. O'Keeffe, O. M. Yaghi, *Acc. Chem. Res.* **2001**, *34*, 319–330.
[4] M. Eddaoudi, J. Kim, N. Rosi, D. Vodak, J. Wachter, M. O'Keeffe, O. M. Yaghi, *Science* **2002**, *295*, 469–472.
[5] B. Moulton, M. J. Zaworotko, *Chem. Rev.* **2001**, *101*, 1629–1658.
[6] J. S. Seo, D. Whang, H. Lee, S. I. Jun, J. Oh, Y. J. Jeon, K. Kim, *Nature* **2000**, *404*, 982.
[7] S. Noro, S. Kitagawa, M. Kondo, K. Seki, *Angew. Chem.* **2000**, *112*, 2162–2164; *Angew. Chem. Int. Ed.* **2000**, *39*, 2082–2084.
[8] S. Kitagawa, M. Kondo, *Bull. Chem. Soc. Jpn.* **1998**, *71*, 1739–1753.
[9] R. Robson, *J. Chem. Soc. Dalton Trans.* **2000**, *21*, 3735–3744.
[10] A. J. Fletcher, E. J. Cussen, T. J. Prior, M. J. Rosseinsky, C. J. Kepert, K. M. Thomas, *J. Am. Chem. Soc.* **2001**, *123*, 10001–10011.
[11] D. V. Soldatov, J. A. Ripmeester, S. I. Shergina, I. E. Sokolov, A. S. Zanina, S. A. Gromilov, Y. A. Dyadin, *J. Am. Chem. Soc.* **1999**, *121*, 4179–4188.
[12] L. C. Tabares, J. A. R. Navarro, J. M. Salas, *J. Am. Chem. Soc.* **2001**, *123*, 383–387.
[13] M. Albrecht, M. Lutz, A. L. Spek, G. van Koten, *Nature* **2000**, *406*, 970–974.
[14] K. Seki, *Phys. Chem. Chem. Phys.* **2002**, *4*, 1968–1972.
[15] D. Li, K. Kaneko, *Chem. Phys. Lett.* **2001**, *335*, 50–56.
[16] R. Kitaura, K. Fujimoto, S. Noro, M. Kondo, S. Kitagawa, *Angew. Chem.* **2002**, *114*, 141–143; *Angew. Chem. Int. Ed.* **2002**, *41*, 133–135.

Glycopeptide Synthesis

Toward Fully Synthetic *N*-Linked Glycoproteins**

*Justin S. Miller, Vadim Y. Dudkin, Gholson J. Lyon, Tom W. Muir, and Samuel J. Danishefsky**

The structural and biological consequences of cellular protein modification through posttranslational glycosylation are central issues in the rapidly growing field of glycobiology.[1] The availability of homogeneous glycopeptides, both *O*-linked (serine, threonine, or tyrosine α-glycosides) and *N*-linked (asparagine β-glycosides), could greatly enhance insight into glycobiology.[2] It became our view that the prospect of total synthesis of homogeneous glycoproteins provides the best chance for gaining such access.

Numerous methods exist for glycopeptide synthesis: glycans have been introduced into peptides by means of amino acid "cassettes" with pendant protected saccharides,[3] through enzymatic manipulations of glycopeptides,[4] or by conjugation of fully elaborated, complex saccharides to short synthetic peptides.[5] Larger *O*-linked glycopeptides have been synthesized by using ligation techniques[6] such as expressed protein ligation.[7] Bertozzi and co-workers extended the scope of the "cassette" approach by applying native chemical ligation to the synthesis of a biologically active glycoprotein with two single-residue *O*-linked glycans.[8] Tolbert and Wong described the ligation of a 392-residue intein-generated peptide thioester and a dipeptide functionalized with a single *N*-acetylglucosamine residue.[7c] Using a different fragment condensation protocol, Hojo et al. reported the synthesis of a glycopeptide domain of Emmprin that contains an *N*-linked chitobiose unit, but the saccharide was not entirely stable to the conditions required for resin cleavage in their solid-phase synthesis.[9]

[*] Prof. S. J. Danishefsky, Dr. J. S. Miller, Dr. V. Y. Dudkin
Laboratory for Bioorganic Chemistry
Sloan-Kettering Institute for Cancer Research
1275 York Avenue, New York, NY 10021 (USA)
Fax: (+1) 212-772-8691
E-mail: s-danishefsky@ski.mskcc.org
Prof. S. J. Danishefsky
Department of Chemistry, Columbia University
Havemeyer Hall, New York, NY 10027 (USA)
G. J. Lyon, Prof. T. W. Muir
Laboratory of Synthetic Protein Chemistry
The Rockefeller University
1230 York Avenue, New York, NY 10021 (USA)

[**] This work was supported by the NIH (AI16943). The receipt of a Pfizer Award to S.J.D. for Creative Work in Organic Synthesis is gratefully acknowledged. We thank Drs. Andrzej Zatorski and Ulrich Iserloh for the preparation of starting materials and for helpful discussions, and Dr. George Sukenick and Ms. Sylvi Rusli (NMR Core Facility, CA-02848) for mass spectral analyses.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author. Experimental details include the preparation of and mass spectral characteristics for **2–6**, **8**, and **10–12**; and NMR spectra for **5**, **6**, **8**, and **10**.

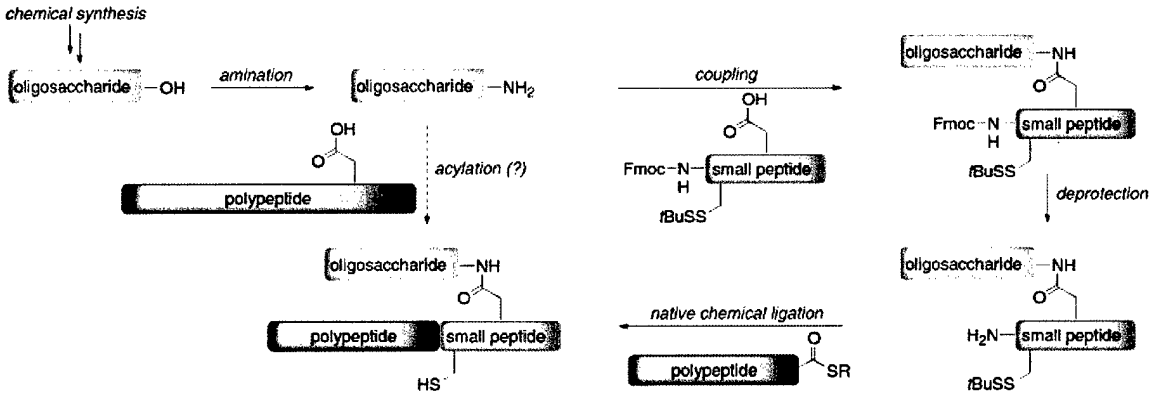
  1433-7851/03/4204-0431 $ 20.00+.50/0

Our goal was to set the stage for fully synthetic routes to complex glycoproteins. It would thus be necessary to harmonize *all* of the components of the undertaking. This includes building a complex glycodomain and incorporating it into a polypeptide setting. Herein we show how the pieces of the puzzle can be interfaced. In launching our program we took particular note of the work of Kochetkov and co-workers,[10] applied by Lansbury and co-workers,[5b] involving direct anomeric β-amination of unprotected saccharides followed by acylation with a peptide carboxylic acid.

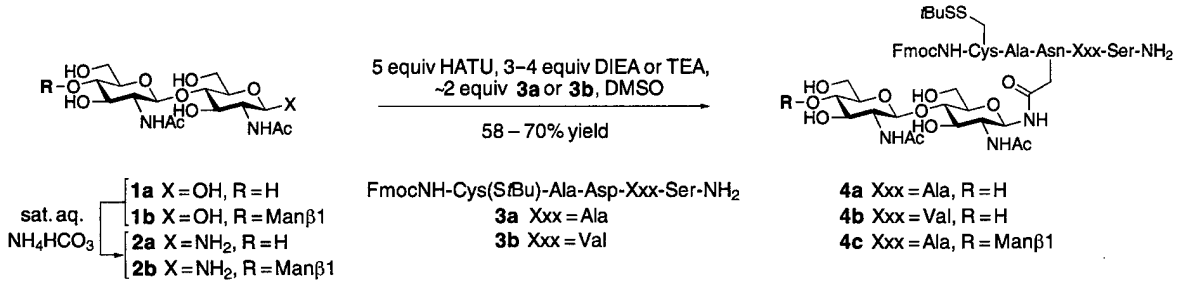
Our program focuses on natural *O*- and *N*-linkages as opposed to non-natural arrangements. Furthermore, as our oligosaccharides are assembled by total chemical synthesis,[11] there is, in principle, no limit to the structural complexity of the carbohydrate sectors of our glycopeptide targets, even as homogeneity is maintained.

The scenario for an ultimately convergent protocol involves merging fully mature oligosaccharide and polypeptide domains in one grand acylation event (dashed arrow, Scheme 1). For the moment, we favored the slightly less convergent, but in the end more practical and certainly more flexible, route shown in Scheme 1. In this case, the anomeric amine function of an oligosaccharide domain is acylated with a more manageable small peptide; native chemical ligation is then used to anneal this construct to a larger polypeptide segment. Critical for our long-term goals was the requirement that the precious, fully synthetic glycan be the limiting reagent in the chemical mergers. As shown in Scheme 1, this boundary condition has been attained.

We began our investigation as shown in Scheme 2. Treatment of known[12] unprotected saccharides **1a–b** (generated by total synthesis) with saturated aqueous ammonium hydrogen carbonate followed by lyophilization to a constant mass afforded glycosylamines **2a–b**. As a consequence of the known instability of anomeric glycosylamines and our desire to maximize yields, the resulting white powders were used without further purification or analysis, aside from mass spectrometry. The results of Kochetkov amination are well-documented,[13] and could in any case be confirmed by ^{1}H NMR coupling constants after peptide conjugation. Using optimized conditions developed for this purpose, glycosylamines **2a–b** were acylated with pentapeptides **3a** or **3b** by adding to the glycosylamine a twofold excess of peptide preactivated with HATU (5 equiv) and a tertiary amine (3–4 equiv) in DMSO. Upon completion of the reactions after only 2–4 h as monitored by analytical HPLC or LCMS, the reaction mixtures were purified by semipreparative HPLC. Two major side products were observed, showing molecular ions of 1 Da and 18 Da less than the starting aspartate-containing peptides. These are consistent with conversion of Asp into Asn through acylation of spurious ammonia and aspartimide formation as shown in Scheme 3,[14] which several other authors also note and seek to avoid by various methods. Though these processes are competitive with glycopeptide formation in terms of rate, their products are solely peptide-derived. Thus an excess of peptide starting material avoids most losses caused by these processes, even with an Ala residue immediately towards the C-terminus of the activated

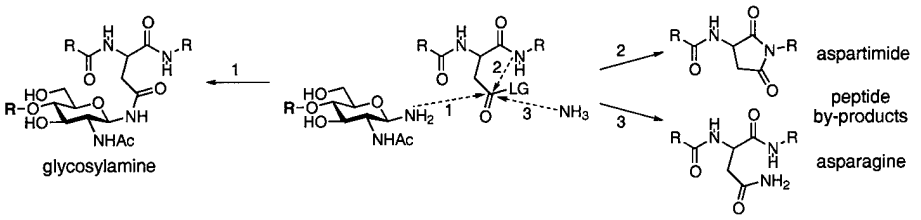


Scheme 1. Convergent approach to *N*-linked glycopeptides. Fmoc = 9-fluorenylmethoxycarbonyl.



Scheme 2. Glycan preparation and peptide conjugation. HATU = *N*-[(dimethylamino)-1*H*-1,2,3-triazole[4,5-*b*]-pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate, DIEA = diisopropylethylamine, TEA = triethylamine, DMSO = dimethyl sulfoxide.

  1433-7851/03/4204-0432 $ 20.00+.50/0



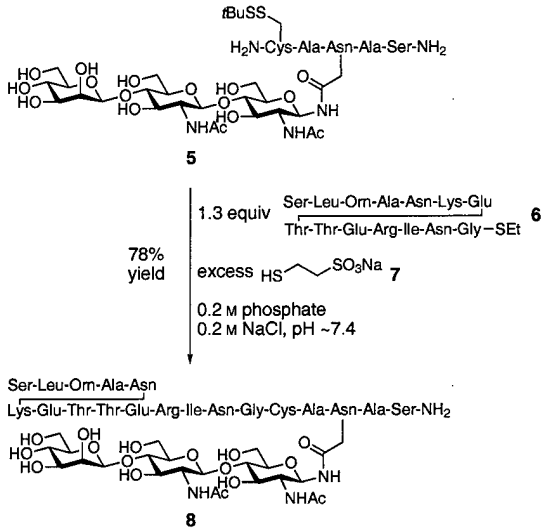
Scheme 3. Peptide byproducts of aspartate acylation.

Asp.[15] The yields of the combined amination and acylation products **4a–c** upon isolation were in the range of 58 to 70% based on starting glycan,[16] representing a significant improvement over the best yields previously reported.

Deprotection of Fmoc-glycopeptide **4c** with 20% piperidine in DMF followed by purification by HPLC afforded free glycopeptide **5** as a cysteine thiol *tert*-butyl disulfide in 68% yield. Additional products were observed with molecular ions identical to that of the desired material, perhaps as a result of epimerization of cysteine or the anomeric amide. The purified, isolated material at this stage was characterized by ^{1}H NMR spectroscopy, electrospray ionization (ES) MS, and liquid chromatography (LC) MS as a single isomer with an anomeric ^{1}H NMR shift ($\delta = 5.04$) and coupling constant ($J =$ 9.6 Hz) indicating the presence of a β-linked anomeric glycosylamide, thus validating the results of the Kochetkov–Lansbury amination.

We next extended glycopeptide **5** through native chemical ligation on a sizable (~15 mg) scale as shown in Scheme 4. As an independent test of the methodology, we synthesized tetradecapeptide thioester **6** employing the Fmoc/*t*Bu solid-phase peptide synthesis method recently reported by Hilvert and co-workers.[17] After automated peptide synthesis on a PEG-type Wang resin,[18] cleavage with trimethylaluminum and ethanethiol in dichloromethane afforded the desired thioester along with several (presumably Glu side chain) thioester derivatives. We noted a significant improvement in the purity of our peptide when the cleavage was quenched by filtration of the cleavage mixture (to remove resin) into a stirred mixture of trifluoroacetic acid, water, and phenol over an ice bath rather than pouring the entire cleavage reaction mixture into the TFA mixture at room temperature; in fact, we observed no side chain thioesters at all when the cleavage was quenched as described.

Ligation of **5** and **6** was carried out in aqueous PBS, 0.2 M in both saline and phosphate, pH ~7.4, in the presence of excess sulfanylethane-2-sulfonate (**7**) as illustrated in Scheme 4. Global disulfide reduction with the water-soluble phosphane TCEP[19] followed by semipreparative HPLC afforded the desired, fully unprotected glycopeptide **8** in 78% yield based on starting glycopeptide. Characterization of glycopeptide **8** by ESMS, LCMS, and ^{1}H and ^{13}C NMR spectroscopy in D_2O (Supporting Information) was consistent with a single compound containing a β-linked glycosylamide.

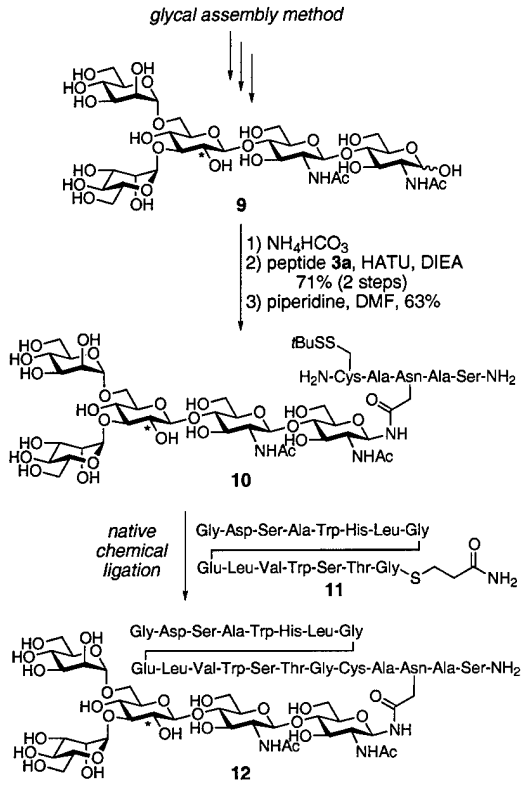


Scheme 4. High-yielding ligation of an *N*-linked glycopeptide.

As an example of the power of this method for complex glycopeptide synthesis, we employed pentasaccharide **9** (Scheme 5), prepared by chemical synthesis.[11] The compound differs from a characteristic high mannose pentasaccharide at one of its 25 stereogenic centers (asterisk, Scheme 5).[20] Amination followed by our peptide acylation conditions with pentapeptide **3a** and Fmoc removal yielded pentasaccharide glycopeptide **10** as a single, β-linked isomer, confirmed by HPLC and ^{1}H NMR spectroscopy ($\delta =$ 5.01 ppm, $J = 9.6$ Hz). Native chemical ligation with **10** and excess pentadecapeptide thioester **11** synthesized by Boc chemistry[21] afforded glycopeptide **12** as evidenced by HPLC and ESMS, again demonstrating proof of principle.

In summary, we have presented a highly convergent route for the production of substantial quantities of homogeneous glycopolypeptides. In this effort we retain the full flexibility accruing from total chemical synthesis of the oligosaccharide (see compound **9**). Of course, the same flexibility is also retained in the polypeptide.[22] Although unexpected difficulties will no doubt be encountered, we presently see no insuperable boundary to progression towards fully synthetic,

  1433-7851/03/4204-0433 $ 20.00+.50/0



Scheme 5. Native chemical ligation of a pentasaccharide glycopeptide and a pentadecapeptide. DMF = *N*,*N*-dimethylformamide.

homogeneous, complex glycoproteins. Applications of the advances recorded above to critical biological goal systems are well underway, and will be described in due course.

Received: September 23, 2002
Revised: November 11, 2002 [Z50217]

[1] a) B. Imperiali, S. E. O'Connor, *Curr. Opin. Chem. Biol.* **1999**, *3*, 643–649; b) B. Imperiali, S. E. O'Connor, T. Hendrickson, C. Kellenberger, *Pure Appl. Chem.* **1999**, *71*, 777–787; c) P. M. Rudd, T. Elliott, P. Cresswell, I. A. Wilson, R. A. Dwek, *Science* **2001**, *291*, 2370–2376; d) J. W. Dennis, M. Granovsky, C. E. Warren, *Biochim. Biophys. Acta* **1999**, *1473*, 21–34; e) J. R. Allen, C. R. Harris, S. J. Danishefsky, *J. Am. Chem. Soc.* **2001**, *123*, 1890–1897.

[2] C. R. Bertozzi, L. L. Kiessling, *Science* **2001**, *291*, 2357–2364.

[3] a) X. T. Chen, D. Sames, S. J. Danishefsky, *J. Am. Chem. Soc.* **1998**, *120*, 7760–7769; b) N. Bezay, G. Dudziak, A. Liese, H. Kunz, *Angew. Chem.* **2001**, *113*, 2350–2353; *Angew. Chem. Int. Ed.* **2001**, *40*, 2292–2295; c) N. Bezay, G. Dudziak, A. Liese, H. Kunz, *Angew. Chem.* **2001**, *113*, 2350–2353; d) J. van Ameijde, H. B. Albada, R. M. J. Liskamp, *J. Chem. Soc. Perkin Trans. 1* **2002**, 1042–1049; e) M. Ciommer, H. Kunz, *Synlett* **1991**, 593–595; f) M. V. Chiesa, R. R. Schmidt, *Eur. J. Org. Chem.* **2000**, 3541–3554; g) E. Meinjohanns, M. Meldal, K. Bock, *Tetrahedron Lett.* **1995**, *36*, 9205–9208.

[4] a) C. Unverzagt, *Tetrahedron Lett.* **1997**, *38*, 5627–5630; b) K. Witte, P. Sears, R. Martin, C. H. Wong, *J. Am. Chem. Soc.* **1997**, *119*, 2114–2118; c) L. X. Wang, M. Tang, T. Suzuki, K. Kitajima, Y. Inoue, S. Inoue, J. Q. Fan, Y. C. Lee, *J. Am. Chem. Soc.* **1997**, *119*, 11137–11146; d) G. Arsequell, G. Valencia, *Tetrahedron: Asymmetry* **1999**, *10*, 3045–3094; e) M. Mizuno, K. Haneda, R. Iguchi, I. Muramoto, T. Kawakami, S. Aimoto, K. Yamamoto, T. Inazu, *J. Am. Chem. Soc.* **1999**, *121*, 284–290; f) K. M. Koeller, M. E. B. Smith, R. F. Huang, C. H. Wong, *J. Am. Chem. Soc.* **2000**, *122*, 4241–4242; g) O. Blixt, K. Allin, L. Pereira, A. Datta, J. C. Paulson, *J. Am. Chem. Soc.* **2002**, *124*, 5739–5746.

[5] a) S. T. Anisfeld, P. T. Lansbury, *J. Org. Chem.* **1990**, *55*, 5560–5562; b) S. T. Cohen-Anisfeld, P. T. Lansbury, *J. Am. Chem. Soc.* **1993**, *115*, 10531–10537; c) E. Meinjohanns, M. Meldal, H. Paulsen, R. A. Dwek, K. Bock, *J. Chem. Soc. Perkin Trans. 1* **1998**, 549–560.

[6] a) T. Wieland, *Chimia* **1974**, *28*, 496–499; b) P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. H. Kent, *Science* **1994**, *266*, 776–779; c) C. F. Liu, J. P. Tam, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 6584–6588.

[7] a) T. W. Muir, D. Sondhi, P. A. Cole, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 6705–6710; b) D. Macmillan, C. R. Bertozzi, *Tetrahedron* **2000**, *56*, 9515–9525; c) T. J. Tolbert, C. H. Wong, *J. Am. Chem. Soc.* **2000**, *122*, 5421–5428.

[8] Y. Shin, K. A. Winans, B. J. Backes, S. B. H. Kent, J. A. Ellman, C. R. Bertozzi, *J. Am. Chem. Soc.* **1999**, *121*, 11684–11689.

[9] H. Hojo, J. Watabe, Y. Nakahara, Y. Ito, K. Nabeshima, B. P. Toole, *Tetrahedron Lett.* **2001**, *42*, 3001–3004.

[10] L. M. Likhosherstov, O. S. Novikova, V. A. Derevitskaja, N. K. Kochetkov, *Carbohydr. Res.* **1986**, *146*, C1–C5.

[11] S. J. Danishefsky, S. Hu, P. F. Cirillo, M. Eckhardt, P. H. Seeberger, *Chem. Eur. J.* **1997**, *3*, 1617–1628.

[12] a) T. Usui, M. Suzuki, T. Sato, H. Kawagishi, K. Adachi, H. Sano, *Glycoconjugate J.* **1994**, *11*, 105–110; b) G. M. Watt, L. Revers, M. C. Webberley, I. B. H. Wilson, S. L. Flitsch, *Angew. Chem.* **1997**, *109*, 2445–2447; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 2354–2356.

[13] D. Vetter, M. A. Gallop, *Bioconjugate Chem.* **1995**, *6*, 316–318.

[14] M. Bodanszky, S. Natarajan, *J. Org. Chem.* **1975**, *40*, 2495–2499.

[15] See Supporting Information for details.

[16] Though aspartimides are prone to open at either imide carbonyl center, it is extremely unlikely that such a side reaction could account for β-peptide product corresponding to more than half of the starting glycosylamine. That the isolated yield here reflects a majority of the starting glycosylamine countermands the possibility that the product derives from nucleophilic (glycosylamine) aspartimide opening.

[17] a) D. Swinnen, D. Hilvert, *Org. Lett.* **2000**, *2*, 2439–2442; b) A. Sewing, D. Hilvert, *Angew. Chem.* **2001**, *113*, 3503–3505; *Angew. Chem. Int. Ed.* **2001**, *40*, 3395–3396.

[18] Solid-phase synthesis of this peptide met with difficulties that were overcome by using a pseudoproline dipeptide monomer; see Supporting Information for details.

[19] J. A. Burns, J. C. Butler, J. Moran, G. M. Whitesides, *J. Org. Chem.* **1991**, *56*, 2648–2650.

[20] Chemical synthesis of glycopeptides offers the ability to introduce structural modifications for the purpose of understanding the role of stereochemistry in glycoconjugate recognition. Evaluation of such stereochemical issues will be reported in due course. The implications of such a point mutation on binding to high mannose lectins is but one example of a fascinating question that can now be answered.

[21] See Supporting Information.

[22] a) B. L. Nilsson, L. L. Kiessling, R. T. Raines, *Org. Lett.* **2000**, *2*, 1939–1941; b) E. Saxon, J. I. Armstrong, C. R. Bertozzi, *Org. Lett.* **2000**, *2*, 2141–2143; c) J. P. Tam, J. X. Xu, K. D. Eom, *Biopolymers* **2001**, *60*, 194–205.

  1433-7851/03/4204-0434 $ 20.00+.50/0

Pergamon

SCIENCE DIRECT®

Tetrahedron Letters 44 (2003) 1791–1793

TETRAHEDRON LETTERS

A concise route to the core pentasaccharide of N-linked glycoproteins

Vadim Y. Dudkin,[a,*] Justin S. Miller[a] and Samuel J. Danishefsky[a,b,*]

[a]*Laboratory for Bioorganic Chemistry, The Sloan-Kettering Institute for Cancer Research, 1275 York Avenue, New York, NY 10021, USA*

[b]*Department of Chemistry, Columbia University, Havemeyer Hall, New York, NY 10027, USA*

Received 12 December 2002; revised 9 January 2003; accepted 10 January 2003

Abstract—A concise preparation of the common pentasaccharide core of the N-linked glycoproteins is described. The reducing end glycal is functionalized at the level of chitobiose, which is then β-mannosylated using Crich's direct coupling protocol. Deprotection of the branching mannose residue, and di-α-mannosylation complete the synthesis.

Our laboratory is currently developing a major program in the total synthesis of glycopolypeptides. Such targets can potentially be used in the development of new diagnostic assays and synthetic vaccines.[1,2] Thus, the preparation of glycopolypeptides is no longer viewed from the primary perspective of a purely academic challenge. While the challenges are indeed daunting, possibilities for building constructs which carry medically valuable information abound with the increasing maturity of the dynamic field of glycobiology.[3] At the present time, chemical synthesis is arguably the only viable way to prepare significant quantities of homogeneous samples of complex glycopeptide targets where the structure of the oligosaccharide can be varied at will.

The abundance of N-linked glycoproteins in nature and the important role they play in cellular interactions prompt major interest in their synthesis by chemical means.[4] It has also been one of the focal research goals of this laboratory.[5] The glycal assembly method[6] has proven itself to be applicable in the preparation of the 'symmetrical' N-linked glycans.[7] Indeed two years ago we reported a route to the signature core region of N-linked glycans.[8,9] In that synthesis a β-linked glucose was introduced at C4 of the chitobiose. Subsequently, epimerization at C2 of the glucose was accomplished by oxidation to a 2-ketoderivative followed by reduction to produce the interior mannose. While this method did provide the core pentasaccharide, the synthesis lacked the directness required to service our growing program. Fortunately, recent developments in carbohydrate synthesis now allow for major simplifications in the elaboration of the core region saccharides. In particular, triflate-mediated direct β-mannosylation methodology, developed by Crich and associates,[10–12] was envisioned for the formation of the key β-mannosyl–chitobiose linkage.[13,14] The Kochetkov amination reaction[15] followed by the Lansbury peptide conjugation procedure[16] enables access to glycopeptides without reliance on intermediate anomeric azides. Moreover, we have recently discovered that such glycans with all of the hydroxyl groups liberated are readily available from the global deprotection of reducing polybenzylated precursors using sodium in liquid ammonia.[17] Figure 1 expresses globally our new synthetic approach taking the advantage of the above findings.

We started with the known disaccharide **1**,[7] which is available from 3,6-di-*O*-benzylglycal.[18] In order to simplify the final steps of the synthesis, it was preferable to

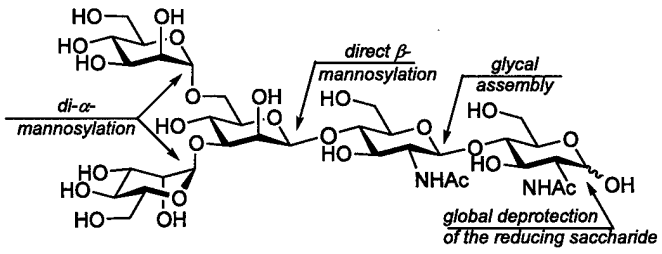


Figure 1.

* Corresponding authors.

0040-4039/03/$ - see front matter
PII: S0040-4039(03)00101-1

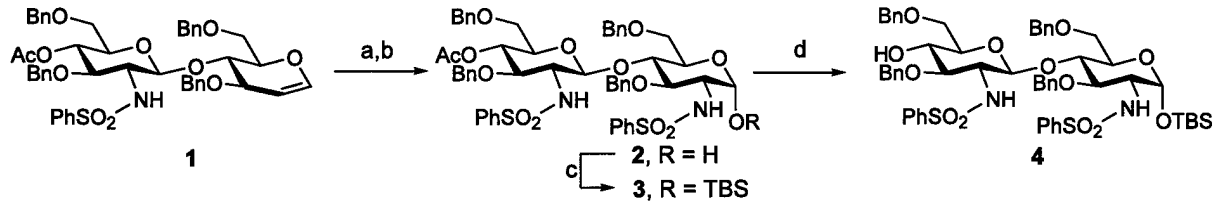
functionalize the reducing end double bond at an early stage. Towards this end, glycal **1** was subjected to iodosulfonamidation (Scheme 1).[19] Following exposure of the addition products to hydrolysis in THF, both isomeric iodosulfonamides were cleanly converted into the reducing disaccharide **2**. Interestingly, the anomeric hydroxyl group in **2** adopts exclusively the α configuration, as evidenced by ^{1}H NMR measurements. This finding can be explained by the intramolecular hydrogen bonding stabilization of the α anomer. Furthermore, this anomeric configuration can be preserved during silylation with excess TBSOTf which gives **3** as a clean α anomer. Deacetylation of **3** following the Zemplén protocol afforded the disaccharide acceptor **4** in 53–60% for the four steps starting from **1**.

Happily, triflate-mediated mannosylation of **4** (Scheme 2) was highly efficient.[20] The coupling reaction, performed at −78°C with 1.5 equiv. of **4** in dichloromethane following the activation of the sulfoxide **5**[21] with triflic anhydride, afforded the mixture of anomers in 85–91% yield and 8/1 β/α ratio.[22] Although an excess of glycosyl acceptor is normally employed in this type of coupling in order to achieve better diastereomeric ratios, the unreacted acceptor is recovered and can be recycled. The isomeric trisaccharides were separated following the removal of the *p*-methoxybenzyl group from mannose 3-OH using ceric ammonium nitrate in wet acetonitrile, providing alcohol **7** as a single anomer in 74% yield.

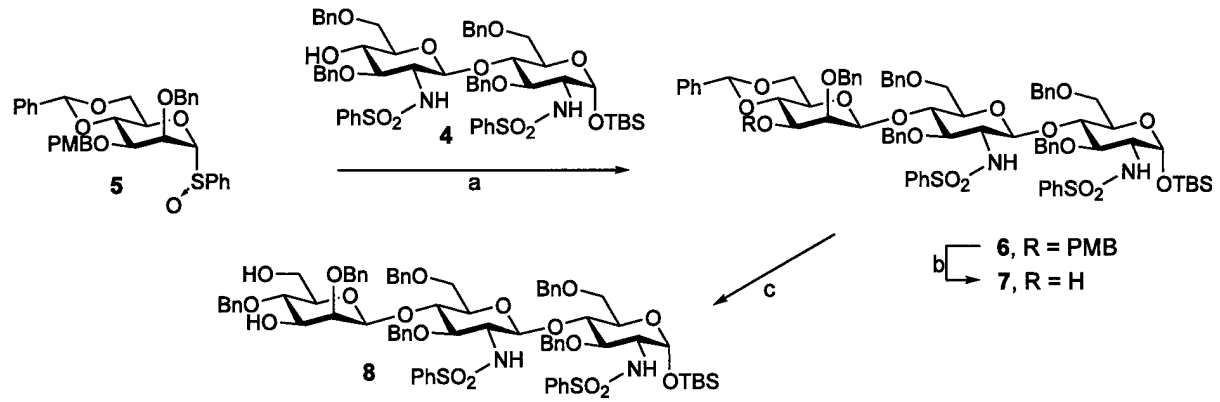
Regioselective cleavage of the 4,6-benzylidene ring was achieved by borane reduction in the presence of dibutylboron triflate.[23] Addition of 3 equiv. of Lewis acid was required to complete the conversion of **7** into the desired 3,6-diol **8**. Simultaneous glycosylation of the primary and secondary hydroxyl groups in **8** was then necessary to form the desired pentasaccharide. Several donors containing ester protection at mannose O2 were screened using various coupling conditions. Glycosylation of **8** with an excess of thiomannoside **9** in acetonitrile using Sinaÿ radical cation activation[24,25] was the most convenient, and provided di-α-mannoside **10** in good yield and complete anomeric selectivity (Scheme 3).

No indication of orthoester formation was found as pentasaccharide **10** was readily saponified producing 'symmetrical' diol **11**. The C2 hydroxyls of the terminal mannose residues in **11** serve as attachments points for lactosamine spacer units, enabling it to be used in the preparation of complex-type glycopeptides. On the other hand, anomeric silyl protection can easily be removed using tetrabutylammonium fluoride to give reducing pentasaccharide **12**.[26]

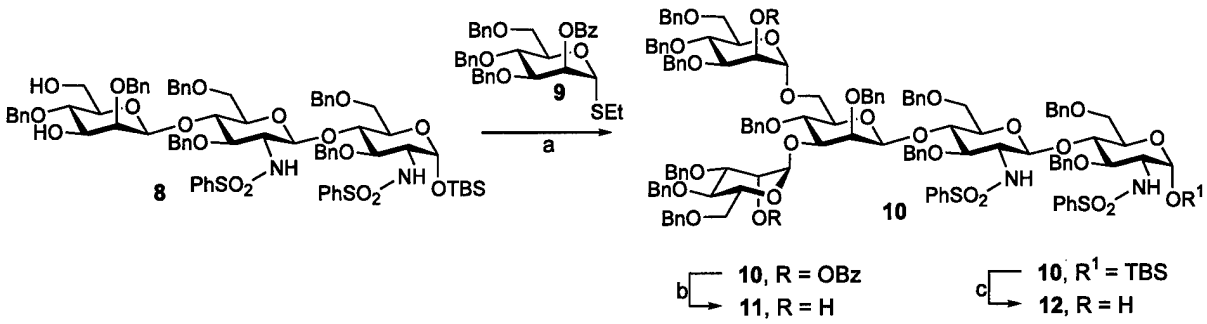
Compound **12**, now reachable in a robust way, serves as a flexible building block in a variety of constructions of highly complex and potentially valuable N-linked glycans. Such extensions and applications of the chemistry described above will be reported in due course.



Scheme 1. *Reagents and conditions*: (a) $I(coll)_2ClO_4$, $PhSO_2NH_2$; (b) Et_3N, H_2O/THF; (c) TBSOTf, 2,6-lutidine, CH_2Cl_2; (d) NaOMe/MeOH; 53–60% for four steps.



Scheme 2. *Reagents and conditions*: (a) i. Tf_2O, DTBMP, CH_2Cl_2, −78°C, ii. **4**, 85–91% (β/α = 8/1); (b) CAN, MeCN/H_2O, 74%; (c) Bu_2BOTf, BH_3·THF, THF, 72%.



Scheme 3. *Reagents and conditions*: (a) $(BrC_6H_4)_3NSbCl_6$, MeCN, 74%; (b) NaOMe/MeOH, 89%; (c) TBAF/AcOH, THF, 81%.

Acknowledgements

This work was supported by the NIH (AI16943). We thank Drs. Andrzej Zatorski and Ulrich Iserloh for the preparation of starting materials and for helpful discussions, and Dr. George Sukenick and Ms. Sylvi Rusli (NMR Core Facility, CA-02848) for mass spectral analyses.

References

1. Danishefsky, S. J.; Allen, J. R. *Angew. Chem., Int. Ed.* **2000**, *39*, 836–863.
2. Kudryashov, V.; Glunz, P. W.; Williams, L. J.; Hintermann, S.; Danishefsky, S. J.; Lloyd, K. O. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 3264–3269.
3. Bertozzi, C. R.; Kiessling, L. L. *Science* **2001**, *291*, 2357–2364.
4. Davis, B. G. *Chem. Rev.* **2002**, *102*, 579–602.
5. Miller, J. M.; Dudkin, V. Y.; Lyon, G. H.; Muir, T. W.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2003**, *42*, in press.
6. Danishefsky, S. J.; Bilodeau, M. T. *Angew. Chem., Int. Ed.* **1996**, *35*, 1380–1419.
7. Danishefsky, S. J.; Hu, S.; Cirillo, P. F.; Eckhardt, M.; Seeberger, P. H. *Chem. Eur. J.* **1997**, *3*, 1617–1628.
8. Wang, Z. G.; Zhang, X. F.; Visser, M.; Live, D.; Zatorski, A.; Iserloh, U.; Lloyd, K. O.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2001**, *40*, 1728–1732.
9. Wang, Z. G.; Zhang, X. F.; Live, D.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2000**, *39*, 3652–3656.
10. Crich, D.; Sun, S. X. *J. Org. Chem.* **1997**, *62*, 1198–1199.
11. Crich, D.; Sun, S. X. *Tetrahedron* **1998**, *54*, 8321–8348.
12. Crich, D.; Sun, S. X. *J. Am. Chem. Soc.* **1998**, *120*, 435–436.
13. Crich, D.; Dudkin, V. *J. Am. Chem. Soc.* **2001**, *123*, 6819–6825.
14. See preceding article.
15. Likhosherstov, L. M.; Novikova, O. S.; Derevitskaja, V. A.; Kochetkov, N. K. *Carbohydr. Res.* **1986**, *146*, C1–C5.
16. Cohen-Anisfeld, S. T.; Lansbury, P. T. *J. Am. Chem. Soc.* **1993**, *115*, 10531–10537.
17. Iserloh, U.; Dudkin, V.; Wang, Z. G.; Danishefsky, S. J. *Tetrahedron Lett.* **2002**, *43*, 7027–7030.
18. Seeberger, P. H.; Cirillo, P. F.; Hu, S. H.; Beebe, X.; Bilodeau, M. T.; Danishefsky, S. J. *Enantiomer* **1996**, *1*, 311–323.
19. Griffith, D. A.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1990**, *112*, 5811–5819.
20. Dudkin, V. Y.; Crich, D. *Tetrahedron Lett.* **2003**, *44*, 1787–1789.
21. Crich, D.; Li, H. M.; Yao, Q. J.; Wink, D. J.; Sommer, R. D.; Rheingold, A. L. *J. Am. Chem. Soc.* **2001**, *123*, 5826–5828.
22. For the use of sulfoxides in glycosylations, see: (a) Kahne, D.; Walker, S.; Cheng, Y.; Engen, D. V. *J. Am. Chem. Soc.* **1989**, *111*, 6881–6882; Gildersleeve, J.; Pascal, R. A.; Kahne, D. *J. Am. Chem. Soc.* **1998**, *120*, 5961–5969.
23. Jiang, L.; Chan, T. H. *Tetrahedron Lett.* **1998**, *39*, 355–358.
24. Zhang, Y. M.; Mallet, J. M.; Sinay, P. *Carbohydr. Res.* **1992**, *236*, 73–88.
25. Marra, A.; Mallet, J. M.; Amatore, C.; Sinay, P. *Synlett* **1990**, 572–574.
26. All new compounds have been characterized by NMR, ESI-MS, and optical rotation.

J|A|C|S
COMMUNICATIONS

Published on Web 12/24/2003

Chemical Synthesis of Normal and Transformed PSA Glycopeptides

Vadim Y. Dudkin,† Justin S. Miller,† and Samuel J. Danishefsky*,†,‡

Laboratory for Bioorganic Chemistry, The Sloan-Kettering Institute for Cancer Research, 1275 York Avenue, New York, New York 10021, and Department of Chemistry, Columbia University, Havemeyer Hall, New York, New York 10027

Received August 18, 2003; E-mail: s-danishefsky@ski.mskcc.org

Localized cancer of the prostate gland can often be arrested, whereas progression to the metastatic state dramatically decreases quality of life and survival rates. The feasibility of early diagnosis of prostate tumors was enhanced with the identification of prostate specific antigen (PSA) as a cancer screening marker.[1−3] PSA, which is a glycoprotein secreted by the prostatic epithelium, manifests high tissue specificity.[4] It consists of 237 amino acid residues and possesses a single *N*-glycosylation site that typically carries an *N*-acetyllactosamine type glycan.[4] Despite the microheterogeneity of normal PSA, its carbohydrates appear to be of the dibranched type (e.g., **1**, Figure 1).[4,5] By contrast, glycans isolated from LnCaP prostatic cancer cells include tri- and even tetrabranched structures (e.g., **2** and **3**, Figure 1).[6] Since the distinctions between normal and "transformed" PSA are limited to glycan composition, they are invisible to current assays which employ antibodies that recognize the glycoprotein's conserved polypeptide domain.[7] Unfortunately, even state-of-the-art diagnostic methods based on PSA levels may fail to distinguish between pre-metastatic prostate cancer and benign prostatic hyperplasia.[8,9] Clinical measurements of PSA levels do not necessarily identify isoforms specific to malignant tissue.[10] This issue is often resolved through invasive biopsy procedures.

We envisioned that differentiated antibodies, sensitive to particular PSA glycoforms, could well form the basis of a new and potentially highly efficient diagnostic strategy to monitor not only the levels of PSA but also the likely aggressiveness of the disease. Furthermore, sensitive screening might enable the pinpointing of malignant transformations at an early stage of the disease, when serum PSA levels are particularly uninformative.

For such antibodies to be elicited, a source of defined and homogeneous PSA fragments bearing *N*-glycans with various degrees of branching is crucial. Challenging as it surely would be, it seemed to us that chemical synthesis might provide the best and most versatile solution to this need. To deal with the complexity of the targets, we hoped to chart new strategies for oligosaccharide assembly, stressing utmost convergency and stereochemical control. We report herein the first chemical synthesis of multibranched *N*-acetyllactosamine-type glycans and their incorporation into PSA glycopeptide fragments **1**−**3**.

In this introductory study, we selected the most common of the multibranched, *N*-acetyllactosamine-type PSA glycans as our targets.[4] Also, we chose not to prepare sialylated forms of the glycans, since these add significantly to the heterogeneiety of serum PSA.[11] Indeed, in the setting of diagnostic assays, samples are first subjected to sialidase digestion.

Earlier, we had found, in simple models, that a sequence consisting of Kochetkov amination[12] of an oligosaccharide bearing a free reducing end, followed by Lansbury aspartylation[13] and

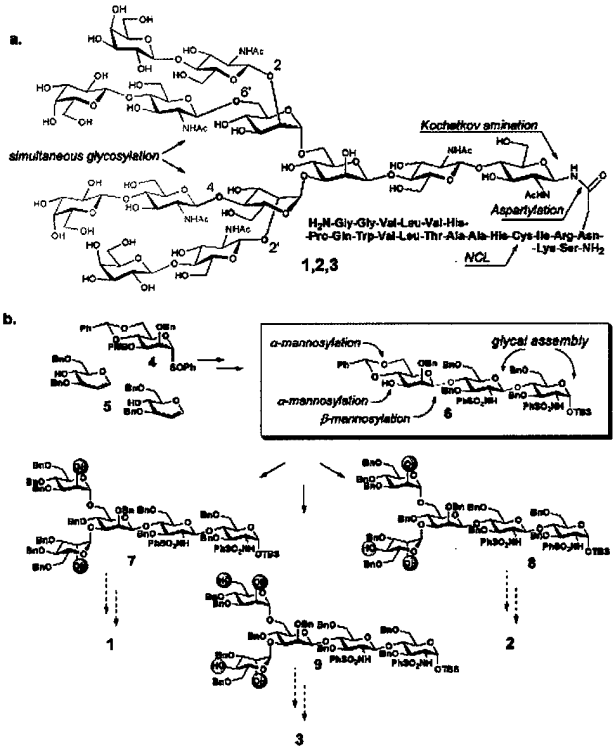


Figure 1. Structures of PSA$^{27-47}$ glycopeptides **1**−**3** (a) and their retrosynthetic analyses (b). **1**, "normal" dibranched PSA fragment with *N*-acetyllactosamines at 2,2′ shown in blue; **2**, tribranched at 2,4,2′ positions (additional orange branch); **3**, tetrabranched at 2,4,2′,6′ (additional orange and red branches).

thence by native chemical ligation[14,15] (NCL), provides a way of building complex *N*-linked polypeptides.[16,17] As will be shown, these protocols served us well in a highly complex setting.

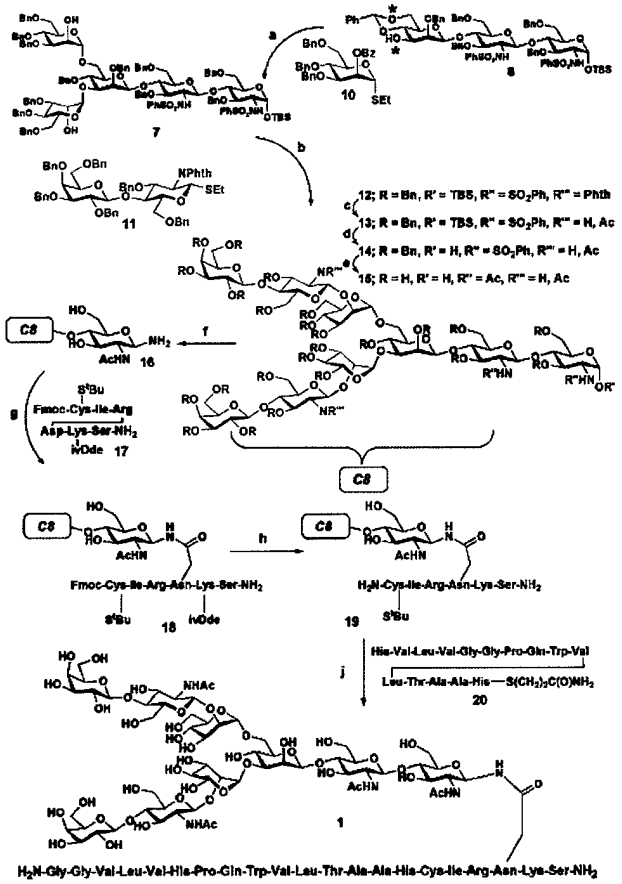
The nexus of the problem, of course, centered around the carbohydrate domains featuring interwoven high mannose and lactosamine blocks. To solve the transformed PSA glycan construction problem, it would be necessary to go well beyond the preparation of symmetrically dibranched glycans (projecting from the 2 and 2′ positions of wing mannoses of the pentasaccacharide core system). While tribranched glycans isolated from natural sources have been used in glycopeptide preparation,[18] symmetrical dibranched structures represented the limit of previous chemical syntheses.[19−23] We set for ourselves the goal of creating a much more encompassing strategy which would pave the way for reaching larger, more branched and less symmetric constructs from a common intermediate (cf. **6**) with high stereoselection and maximum convergency.[24]

Our approach relied on a proposal that introduction of several *N*-acetyllactosamines can be accomplished in a single glycosylation

† The Sloan-Kettering Institute for Cancer Research.
‡ Columbia University.

10.1021/ja037988s CCC: $27.50

Scheme 1. Synthesis of Normal PSA Glycopeptide **1**[a]

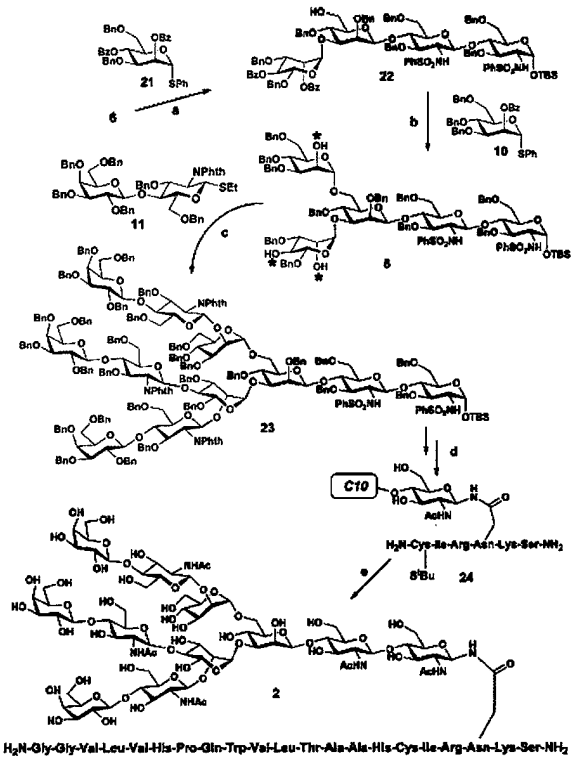


[a] Reagents and conditions: (a) i. BH_3·THF, Bu_2BOTf, THF, 72%; ii. **10**, $(BrC_6H_4)_3NSbCl_6$, MeCN, 74%; iii. NaOMe·MeOH, 89%; (b) MeOTf, DTBP, CH_2Cl_2, 60%; (c) i. ethylenediamine, *n*-BuOH·toluene, 90 °C, ii. Ac_2O/py, iii. NaOMe/MeOH, 72%; (d) TBAF/AcOH, THF, 76%; (e) i. Na/NH_3, −78 °C, ii. Ac_2O, iii. NaOMe/MeOH, 65%; (f) NH_4HCO_3/H_2O; (g) **17**, HATU, Hünig's base, DMSO, 61% from **15**; (h) $(NH_2)_2$, piperidine, DMF, 62%; (j) **20**, MES-Na, pH = 7.4, 17%.

event. This transformation has been demonstrated in a similar setting in simpler models.[25,26] The PSA glycan synthesis problem could then be translated into that of producing pentasaccharides **7**, **8**, and **9** with differentiated "free OH" acceptor sites. This retrosynthesis took us back to trisaccharide **6** as a common intermediate.[27] This key building block contains virtual (see benzylidene acetal) and identified acceptor loci.[28] By α-mannosylation with suitably differentiated α-mannosyl donors, permuted core pentassacharides **7**, **8**, and **9** quickly became accessible. The central intermediate trisaccharide **6** is smoothly assembled by a combination of glycal assembly in the context of sulfonamidoglycosylation and sulfonamidohydroxylation[29] (see **4** → AB rings of **6**) and Crich's β-mannosylation chemistry[30,31] (see **5** → ring C of **6**).[27] Building blocks **4** and **5** are prepared from D-glucal and mannose, respectively.

The validity of the concept was first field-tested in the context of a synthesis of the nontransformed-type glycan **1** (Scheme 1). Thus, trisaccharide **6** was prepared following the logic described above.[27] Reductive cleavage of the benzylidene acetal generated a diol that coupled at two points (see asterisks in **6**) with monoester-containing α-mannosyl donor **10** to assemble a pentasaccharide containing two esters. Cleavage of the two benzoates led to bis acceptor **7**. Indeed, two-fold glycosylation using donor **11** proceeded smoothly to establish the protected core system (**12**) corresponding

Scheme 2. Synthesis of Tribranched Transformed PSA Glycopeptide **2**[a]



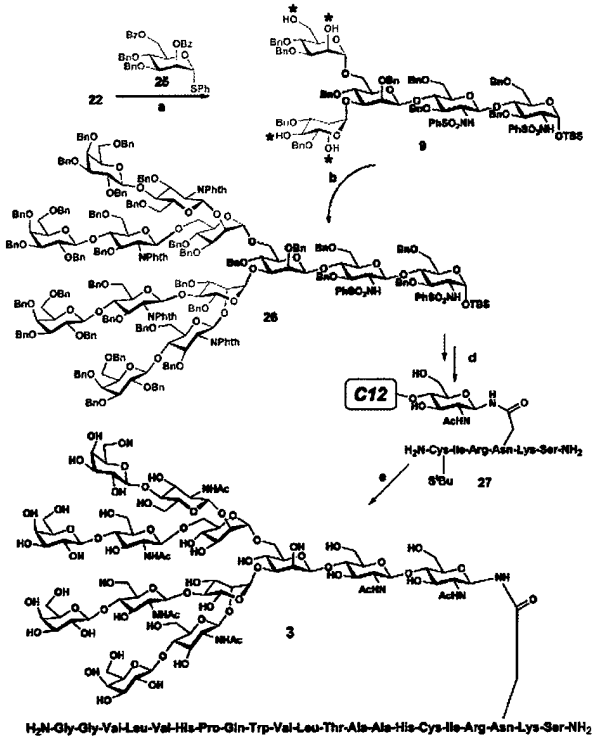
[a] Reagents and conditions.: (a) i. **21**, $(BrC_6H_4)_3NSbCl_6$, MeCN, 71%; ii. BH_3·THF, Bu_2BOTf, THF, 85%; (b) i. **10**, $(BrC_6H_4)_3NSbCl_6$, MeCN, ii. NaOMe/MeOH, 72%; (c) **11**, $(BrC_6H_4)_3NSbCl_6$, MeCN, 41%; (d) see Scheme 1, (c−h), 11% for 10 steps; (e) **20**, MES-Na, pH = 7.4, 38%

to **1**. The two phthalimides were then converted into acetamides, the anomeric hydroxyl group was liberated by desilylation, and the product was subjected to global deprotection (sodium in liquid ammonia). Here, we exploited our remarkable finding that the integrity of the reducing end hemiacetal is maintainable during global Birch debenzylation.[32] Amine-specific diacetylation afforded free glycan **15** as a mixture of anomers.[33] Free β-glycosylamine **16** was obtained from nonasaccharide **15** by a Kochetkov amination protocol. Coupling with excess hexapeptide **17** gave glycoconjugate **18**. The Fmoc and ivDde protecting groups in **18** were shed, and the resulting amine was subjected to NCL with pentadecapeptide thioester **20**. This sequence afforded the fully characterized normal PSA(27−47) glycopeptide fragment, presented as homogeneous nonasaccharide uneicosapeptide **1**.

Having tested our strategy in the control synthesis of **1**, we sought to apply these notions to the syntheses of **2** and **3**, as described in Schemes 2 and 3. The key point to be appreciated is that simple permutations in the processing and advancement of key trisaccharide **6**, and selection of the resident protection patterns in the α-mannosylation donors used in ring extension reactions of the strategic trisaccharide, build high diversity and high complexity at a stage where the systems are still of relatively modest size.

We first turned to the nonsymmetrically branched PSA glycan **2**. Here we extended, sequentially, the hydroxyls at C3 and C6 of the ring C system. Thus, we first accomplished α-mannosylation at C3,[34] using donor **21** bearing two ester linkages, leading to 4,6-benzylidene-protected tetrasaccharide. Controlled reductive cleavage of the benzylidene acetal[35] exposes the C6 hydroxyl of the C ring in **22**, which was α-mannosylated with the previously employed monoester α-mannosyl donor **10**. At this stage, the three esters were

Scheme 3. Synthesis of Tetrabranched Transformed PSA Glycopeptide **3**[a]



[a] Reagents and conditions: (a) i. **25**, $(BrC_6H_4)_3NSbCl_6$, MeCN, 74%, ii. NaOMe/MeOH, 92%; (b) **11**, $(BrC_6H_4)_3NSbCl_6$, MeCN, 19%; (d) see Scheme 1, (c–h), 32%; (e) **20**, MES-Na, pH = 7.4, 65%.

easily cleaved, thereby exposing trivalent acceptor system **8**. Three-fold β-lactosylation was accomplished using β-lactosamine donor **11** with stereodirecting phthalimide groups at C2′. Indeed, three such donors were incorporated, leading to the protected core system (**23**) corresponding to **2**. The steps for progressing from **23** to **2** were much as those worked out in advancing from **12** to **1** (vide supra).

Thus encouraged, we now undertook the challenge of reaching the highly branched system **3**. Toward this end, we revisited tetrasaccharide **22**. Reductive cleavage of the benzylidene acetal, as before, was now followed by mannosylation with **25**, bearing esters at C2′ and C6′. This reaction provided the required pentasaccharide, containing four acceptor sites momentarily masked as benzoate esters. The key hydroxy centers were smoothly unveiled (see **9**). At this stage, four-fold glycosylation was accomplished with lactosamine donor **11**, but this time in more modest yield. The tridecasaccharide core system (cf. **26**) was obtained, but this time in 19% yield.

Fortunately, the sequence from protected oligosaccharide to deprotected Kotchetkov amination product worked well, as did the introduction of **17** via aspartylation and deprotection (cf. **27**). Upon NCL with **19**, the tridecassacharide–uneicosapeptide glycoconjugate **3** was delivered in homogeneous form.

In summary, a universal strategy for the preparation of complex *N*-linked glycopeptides from a common precursor has been developed. This new methodology has proven its mettle in the preparation of normal and transformed PSA fragments. This particular project has now moved on from a purely chemical focus to the immunological realm. The next step involves production of selective antibodies through animal immunization; the results will be reported in due course.

Acknowledgment. This work was supported by the NIH (AI16943/CA10382). We thank Dr. Andrzej Zatorski for the preparation of starting materials and for helpful discussions, and Ms. Anna Dudkina for help with mass spectral analyses and HPLC separations. J.S.M. acknowledges support from the U.S. Army Prostate Cancer Research Program (PC020147). V.Y.D. is a fellow of the U.S. Army Breast Cancer Research Foundation (BC020513).

Supporting Information Available: Experimental procedures and compound characterization data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Armbruster, D. A. *Clin. Chem.* **1993**, *39*, 181–195.
(2) Abrahamsson, P. A.; Lilja, H.; Oesterling, J. E. *Urol. Clin. N. Am.* **1997**, *24*, 353.
(3) Egawa, S. *Biomed. Pharmacother.* **2001**, *55*, 130–134.
(4) Okada, T.; Sato, Y.; Kobayashi, N.; Sumida, K.; Satomura, S.; Matsuura, S.; Takasaki, M.; Endo, T. *Biochim. Biophys. Acta* **2001**, *1525*, 149–160.
(5) Belanger, A.; Vanhalbeek, H.; Graves, H. C. B.; Grandbois, K.; Stamey, T. A.; Huang, L. H.; Poppe, I.; Labrie, F. *Prostate* **1995**, *27*, 187–197.
(6) Prakash, S.; Robbins, P. W. *Glycobiology* **2000**, *10*, 173–176.
(7) Ward, A. M.; Catto, J. W. F.; Hamdy, F. C. *Ann. Clin. Biochem.* **2001**, *38*, 633–651.
(8) Semjonow, A.; Hertle, L. *Urol.-Ausg. A* **1995**, *34*, 290–296.
(9) Semjonow, A.; Brandt, B.; Oberpenning, F.; Roth, S.; Hertle, L. *Prostate* **1996**, 3–16.
(10) Masters, J. G.; Keegan, P. E.; Hildreth, A. J.; Greene, D. R. J. *Br. J. Urol.* **1998**, *81*, 419–423.
(11) Hilz, H.; Noldus, J.; Hammerer, P.; Buck, F.; Luck, M.; Huland, H. *Eur. Urol.* **1999**, *36*, 286–292.
(12) Likhosherstov, L. M.; Novikova, O. S.; Derevitskaja, V. A.; Kochetkov, N. K. *Carbohydr. Res.* **1986**, *146*, C1–C5.
(13) Cohen-Anisfeld, S. T.; Lansbury, P. T. *J. Am. Chem. Soc.* **1993**, *115*, 10531–10537.
(14) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776–779.
(15) Tolbert, T. J.; Wong, C. H. *J. Am. Chem. Soc.* **2000**, *122*, 5421–5428.
(16) Bertozzi, C. R.; Kiessling, L. L. *Science* **2001**, *291*, 2357–2364.
(17) Miller, J. S.; Dudkin, V. Y.; Lyon, G. J.; Muir, T. W.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2003**, *42*, 431–434.
(18) Meinjohanns, E.; Meldal, M.; Paulsen, H.; Dwek, R. A.; Bock, K. *J. Chem. Soc., Perkin Trans. 1* **1998**, 549–560.
(19) Wang, Z. G.; Zhang, X. F.; Visser, M.; Live, D.; Zatorski, A.; Iserloh, U.; Lloyd, K. O.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2001**, *40*, 1728–1732.
(20) Unverzagt, C. *Carbohydr. Res.* **1997**, *305*, 423–431.
(21) Unverzagt, C.; Andre, S.; Seifert, J.; Kojima, S.; Fink, C.; Srikrishna, G.; Freeze, H.; Kayser, K.; Gabius, H. J. *J. Med. Chem.* **2002**, *45*, 478–491.
(22) Prahl, I.; Unverzagt, C. *Angew. Chem., Int. Ed.* **2002**, *41*, 4259–4262.
(23) Seifert, J.; Lergenmuller, M.; Ito, Y. *Angew. Chem., Int. Ed.* **2000**, *39*, 531–534.
(24) During the manuscript revision process, a synthesis of complex-type glycans (not linked to peptides) with bisecting GlcNAc was described: Weiss, H.; Unverzagt, C. *Angew. Chem., Int. Ed.* **2003**, *42*, 4261–4263.
(25) Lönn, H.; Lönngren, J. *Carbohydr. Res.* **1983**, *120*, 17–24.
(26) Matsuzaki, Y.; Ito, Y.; Nakahara, Y.; Ogawa, T. *Tetrahedron Lett.* **1993**, *34*, 1061–1064.
(27) Dudkin, V. Y.; Miller, J. S.; Danishefsky, S. J. *Tetrahedron Lett.* **2003**, *44*, 1791–1793.
(28) Dudkin, V. Y.; Crich, D. *Tetrahedron Lett.* **2003**, *44*, 1787–1789.
(29) Griffith, D. A.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1990**, *112*, 5811–5819.
(30) Crich, D.; Sun, S. X. *J. Am. Chem. Soc.* **1998**, *120*, 435–436.
(31) Crich, D.; Sun, S. *Tetrahedron* **1998**, *54*, 8321–8348.
(32) Iserloh, U.; Dudkin, V.; Wang, Z. G.; Danishefsky, S. J. *Tetrahedron Lett.* **2002**, *43*, 7027–7030.
(33) Calarese, D. A.; Scanlan, C. N.; Zwick, M. B.; Deechongkit, S.; Mimura, Y.; Kunert, R.; Zhu, P.; Wormald, M. R.; Stanfield, R. L.; Roux, K. H.; Kelly, J. W.; Rudd, P. M.; Dwek, R. A.; Katinger, H.; Burton, D. R.; Wilson, I. A. *Science* **2003**, *300*, 2065–2071.
(34) Zhang, Y. M.; Mallet, J. M.; Sinay, P. *Carbohydr. Res.* **1992**, *236*, 73–88.
(35) Jiang, L.; Chan, T. H. *Tetrahedron Lett.* **1998**, *39*, 355–358.

JA037988S